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ROLE OF GPS2 IN EPIGENOME ALTERATIONS LINKED TO METAFLAMMATION

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To my dear parents, my beloved wife and my lovely son!

ABSTRACT

Current evidence suggests that transcriptional and epigenomic reprogramming events, triggered by transcription factors and chromatin-modifying co-regulators, are of central importance for disease development. G-protein pathway suppressor 2 (GPS2) is a key component of the HDAC3 co-repressor complex that has been earlier implicated in cholesterol homeostasis and anti-inflammatory crosstalk. More recent work revealed that the expression and function of GPS2 is altered in obese humans and correlated to the inflammation status and the risk for developing type 2 diabetes. Although this potentially suggests the involvement of GPS2 in metaflammation, i.e. closely linked metabolic and inflammatory disease pathways, the underlying mechanisms and the precise role of GPS2 remained unknown. The aim of this thesis was to characterize the functions of GPS2 at the molecular and physiological level with an emphasis on obesity-associated inflammation, insulin resistance, and fatty liver disease.

In **Paper I**, we identified GPS2 as a key regulator of ABCA1-dependent cholesterol efflux in inflammatory macrophages. This study potentially implicates the GPS2-ABCA1 axis in linking obesity and type 2 diabetes to cardiovascular diseases.

In **Paper II**, we identified GPS2-repressed pro-inflammatory enhancers and deeply characterized enhancer structure and function at the *Ccl2* gene locus in macrophages. The study revealed that GPS2-repressed enhancers are non-redundant and that inhibiting enhancer-transcribed eRNAs reduced gene expression, thus suggesting eRNA function.

In **Paper III**, we characterized macrophage-specific *Gps2* knockout mice along with *in vitro* models and expression analysis in humans to identify a potent anti-inflammatory role of GPS2 and the underlying genomic actions. Upon diet-induced obesity, *Gps2* knockout mice display hallmarks of metaflammation typical for obese humans, i.e. elevated inflammation and insulin resistance.

In **Paper IV**, we describe hitherto unknown liver functions of GPS2 in the development of the non-alcoholic fatty liver disease. Through integrated genomic and phenotypic characterization of hepatocyte-specific *Gps2* knockout mice, we found that GPS2 specifically antagonizes the fatty acid receptor PPARα. Thus, the selective modulation of GPS2-PPARα interactions could be of therapeutic interest for future interventions.

In conclusion, this thesis revealed novel insights into the multifaceted regulatory roles of GPS2 in altering epigenomes and transcription linked to metabolic and inflammatory processes. These insights should help to better understand the development of obesity, type 2 diabetes, atherosclerosis, and fatty liver disease, and they may help to define novel therapeutic strategies.

LIST OF SCIENTIFIC PAPERS

I. **Zhiqiang Huang**, Ning Liang, Anastasius Damdimopoulos, Rongrong Fan, Eckardt Treuter. G-protein pathway suppressor 2 links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages. FASEB Journal, 2018, fj201801123R.

II. **Zhiqiang Huang**, Rongrong Fan, Saioa Goñi, Anastasios Damdimopoulos, Fawaz Alzaid, Raphaelle Ballaire, Tomas Jakobsson, Nicolas Venteclef, Eckardt Treuter. A GPS2-repressed enhancer RNA regulates Ccl2 transcription and triggers obesity-associated inflammation (Manuscript)

III. Rongrong Fan, Amine Toubal, Saioa Goñi, Karima Drareni, **Zhiqiang Huang**, Fawaz Alzaid, Raphaelle Ballaire, Patricia Ancel, Ning Liang, Anastasios Damdimopoulos, Isabelle Hainault, Antoine Soprani, Judith Aron-Wisnewsky, Fabienne Foufelle, Toby Lawrence, Jean-Francois Gautier, Nicolas Venteclef, Eckardt Treuter. Loss of the co-repressor GPS2 sensitizes macrophage activation upon metabolic stress induced by obesity and type 2 diabetes. Nature Medicine, 2016, 22(7):780-91.

IV. Ning Liang, Anastasius Damdimopoulos, Saioa Goñi, **Zhiqiang Huang**, Lise-Lotte Vedin, Tomas Jakobsson, Marco Giudici, Ahmed Osman, Matteo Pedrelli, Serena Barilla, Fawaz Alzaid, Arturo Mendoza, Tarja Schröder, Raoul Kuiper, Paolo Parini, Anthony Hollenberg, Philippe Lefebvre, Bart Staels, Nicolas Venteclef, Eckardt Treuter, Rongrong Fan. GPS2 accelerates the progression of nonalcoholic steatohepatitis through PPARα-selective mechanisms (Manuscript)

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Karima Drareni, Raphaëlle Ballaire, Serena Barilla, Mano J. Mathew, Amine Toubal, Rongrong Fan, Ning Liang, Catherine Chollet, **Zhiqiang Huang**, Maria Kondili, Fabienne Foufelle, Antoine Soprani, Ronan Roussel, Jean-François Gautier, Fawaz Alzaid, Eckardt Treuter, Nicolas Venteclef. GPS2 Deficiency Triggers Maladaptive White Adipose Tissue Expansion in Obesity via HIF1A Activation. Cell Reports. 2018, 24(11): 2957-2971.

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette subfamily G member 1
AP1	Activator protein 1
ATCC	American Type Culture Collection
ATM	Adipose Tissue Macrophages
BMDMs	Mouse bone marrow-derived macrophages
Cas9	CRISPR associated protein 9
CBP	CREB-binding protein
CCL2	C-C motif chemokine ligand 2
CCL7	C-C motif chemokine ligand 7
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chromatin Immunoprecipitation followed by sequencing
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
eRNA	Enhancer-derived non-coding RNAs
epiWAT	Epididymal white adipose tissue
FACS	Fluorescence-Activated Cell Sorting
FGF21	Fibroblast growth factor 21
GPS2	G-protein Pathway Suppressor 2
GRO-seq	Global Run-on sequencing
HDAC3	Histone deacetylase 3
H3K4me3	Histone H3 Lysine 4 trimethylation
H3K27ac	Histone H3 Lysine 27 acetylation
HAT	Histone acetylase
HDL	High-density lipoprotein
HFD	High-Fat Diet
Hi-C	High-through chromosome conformation capture
HMDMs	Human Monocyte-Derived Macrophages
IHC	Immunohistochemistry
IFN	Interferon
ingWAT	Inguinal white adipose tissue
IKK	IκB kinase

IL4	Interleukin-4
IL6	Interleukin 6
IL10	Interleukin 10
IL13	Interleukin 13
IL1β	Interleukin 1 beta
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinases
LDL	Low-density lipoprotein
LDTFs	Lineage-Determining Transcription Factors
LFD	Low-fat diet
LKO	Hepatocyte-specific Gps2 knockout
LPS	Lipopolysaccharide
LNA	Locked Nucleic Acids
lncRNA	long non-coding RNAs
LXRs	Liver X receptors alpha and beta (alias NR1H3, NR1H2)
LXRE	LXR response ellement
MKO	Macrophage-specific Gps2 knockout
mRNA	Messenger RNA
KEGG	Kyoto Encyclopedia of Genes and Genomes
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCOR	Nuclear receptor corepressor 1
NF-κB	Nuclear factor kappa B
NGS	Next-generation sequencing
NO	Nitric oxide
p300	E1A binding protein p300
Pol II	RNA polymerase 2
PPAR	Peroxisome Proliferator Activated Receptor
PTGS1	Prostaglandin-Endoperoxide Synthase 1
PU.1	Transcription factor PU.1
qRT-PCR	Quantitative reverse transcription PCR
RAW cells	Mouse macrophage RAW264.7 cell line
RBP4	Retinol-binding protein 4
RCT	Reverse cholesterol transport
RNA-seq	RNA Sequencing

RNAi	RNA interference
RUNX1	Runt-related transcription factor 1
RXR	Retinoid X receptor
shRNA	Short hairpin RNA
SMRT	Silencing Mediator of Retinoid and Thyroid hormone receptors
STAT	Signal transducer and activator of transcription
T2D	Type 2 Diabetes
TBL1	Transducin-Beta-Like Protein 1
TBLR1	TBL1-Related Protein 1
TEPMs	Mouse thioglycollate-elicited peritoneal macrophages
TF	Transcription factor
Th1	T-cell responses 1
Th2	T-cell responses 2
TLR4	Toll-like receptor 4
TNFα	Tumor Necrosis Factor- α
TSS	Transcription Start Site
VLDL	Very-low-density lipoprotein
WAT	White Adipose Tissue
ω-3	omega-3

1 INTRODUCTION

1.1 Macrophages

Macrophages are the crucial component of innate immunity and play an important role in tissue host defense and homeostasis [1]. Tissue macrophages provide the first defense against microorganisms and protect the body from infection through the release of cytokines [1-5]. Tissue-resident macrophages include brain microglia, lung alveolar macrophages, liver Kupffer cells, spleen macrophages, adipose tissue macrophages, kidney intraglomerular mesangial macrophages and intestinal macrophages, amongst others. They are mainly developed from progenitor cells in the yolk sac or from bone marrow hematopoietic stem cells [6].

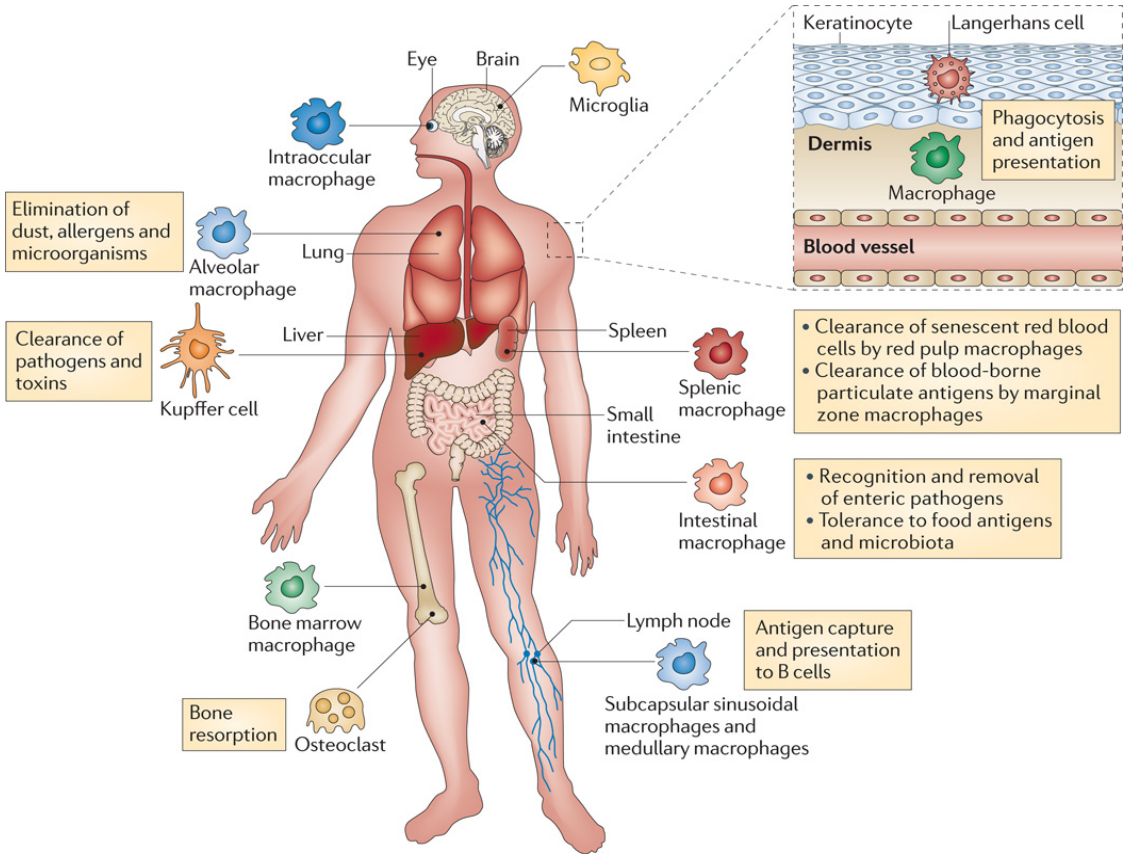

Figure 1. Tissue `imprinting´ generates macrophage heterogeneity.

Reprinted from Nature reviews immunology, Vol. 11, p. 723-737. Peter J. Murray and Thomas A. Wynn, Protective and pathogenic functions of macrophage subsets, doi:10.1038/nri3073, Copyright 2011, with permission from Nature.

The differentiation and maturation of tissue-resident macrophages require precise regulation by a group of growth factors and transcription factors (TFs) such as PU.1 and Runt-related transcription factor 1 (RUNX1) [6-10]. As shown in **Figure 1**, tissue-resident macrophages are functionally different dependent on the tissue-specific signals and the

surrounding microenvironment [9, 10]. Disturbing this process could alter normal macrophage function, leading to diseases such as infections, tumors, metabolic and autoimmunity diseases [10-12].

1.1.1 Macrophage lipid metabolism

As a defensive cell type, macrophages mainly participate in phagocytosis, muscle regeneration, wound healing, limb regeneration, iron homeostasis, lipid metabolism, and pigment retention. All these processes are dynamically balanced to maintain the health status [13-15].

Three major types of pathways in mammals cooperatively regulate the lipid (i.e. triglycerides and cholesterol) metabolism. These include 1) exogenous pathways, 2) endogenous pathways, and 3) reverse cholesterol transport (RCT). The exogenous pathways refer to the process of intestinal uptake of chylomicrons and their transport in the bloodstream to the peripheral tissues where the chylomicrons are further degraded to free fatty acids and cholesterol. In contrast, the endogenous pathways mainly represent the very low-density lipoproteins (VLDLs) synthesis and transport from the liver to the peripheral tissues (mainly adipose tissues), where VLDLs are degraded to free fatty acids and low-density lipoproteins (LDLs) [5, 16]. Then LDLs deliver cholesterol and circulating triglycerides to the liver again. RCT is a complex process resulting in the cholesterol transport from peripheral tissues to the liver in the form of high-density lipoproteins (HDL) [17].

RCT is essential for cell homeostasis as most cells cannot metabolize cholesterol. Macrophages are the critical cells which regulate and metabolize cholesterol through RCT. Macrophages assimilate cholesterol via phagocytosis and macropinocytosis and export the endogenous cholesterol to HDL via the RCT process. Disruption of this process leads to macrophage cholesterol accumulation which generates foam cells, an early risk factor to cause plaque formation and atherosclerosis development [14, 18]. ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) are the major transporters to regulate cholesterol efflux in the macrophages with the help of lipid-poor apoA1 (**Figure 2**). After circulation, the mature HDL transport the cholesteryl esters to the liver in the form of triglycerides, LDL and VLDL. These lipoproteins can be further transferred and secreted by bile acids [5, 17, 18].

ABCA1 and ABCG1 ablation could significantly reduce the RCT both *in vitro* and *in vivo*, which resulted in the development of atherosclerotic lesions [19, 20]. The functional difference between ABCA1 and ABCG1 in RCT is that ABCA1 transports cholesterol to HDL and ABCG1 transports cholesterol to apoA1 [20]. Liver X receptors (LXRα and LXRβ) are the key TF for the regulation of ABCA1/G1 gene expression. LXRs are ligand-dependent TFs which control cholesterol homeostasis in liver and peripheral tissues. LXRs specifically recognize the LXR response element (LXRE) and bind to gene promoters and enhancers in the form of LXR/ Retinoid X receptor (RXR) heterodimers. The endogenous LXR ligands are oxysterols and oxidized cholesterol while the synthetic LXR ligands T0901317 and GW3965 are widely used in research, which makes LXRs potential drug-targetable candidates for the treatment of atherosclerosis and metabolic syndrome [14, 21-23].

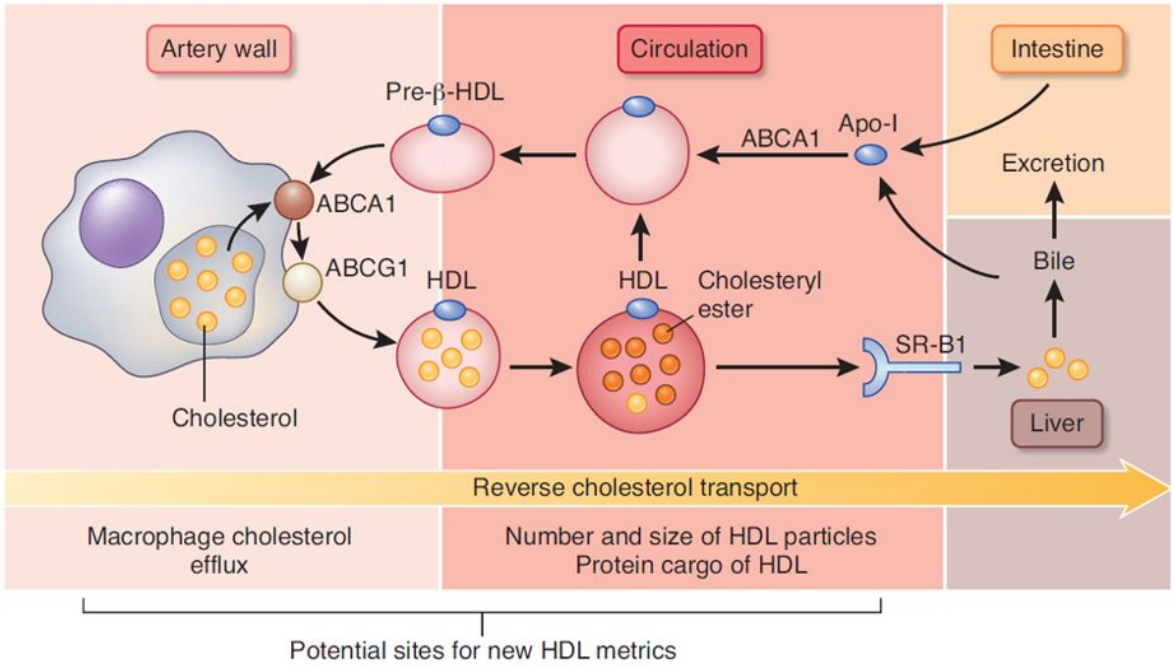

Figure 2. Macrophage-mediated reverse cholesterol transport (RCT).

Reprinted from Nature Medicine, Vol. 18, p.1346-1347. Peter Jay W Heinecke, A new era for quantifying HDL and cardiovascular risk? DOI: 10.1038/nm.2930, Copyright 2012, with permission from Springer Nature.

1.1.2 Macrophage inflammation

The commonly described macrophage polarization states are classical M1 polarization and alternative M2 polarization (**Figure 3**) [24-26]. The classical M1 activation, which can be induced by toll-like receptor 4 (TLR4) ligands, interferon-γ (IFNγ) or tumor necrosis factor-

α (TNFα), is characterized by a high level of antigen presentation, high expression of pro-inflammatory cytokines and chemokines such as C-C motif chemokine ligand 2 (*Ccl2*) and *Tnfα*, high production of nitric oxide (NO) and reactive oxygen intermediates (ROI) [25-27]. In contrast, the alternative M2 activation, which can be induced by interleukin 4/13 (IL4/IL13), is characterized by a high expression of anti-inflammatory cytokines such as interleukin 10 (IL10) and promotes tissue remodeling and repair [27-29]. In mice models, M2 macrophages display high expression of signature genes including found in inflammatory zone 1 (*Fizz1*), arginase (*Arg1*) and chitinase-like protein 3 (*Chil3*). M2 macrophages can be further subdivided into subtypes based on different signaling pathways and chemokine expression [26]. For example, IL4/IL13 signaling pathways induce the M2a macrophages, which have high expression of *Ccl17*, *Ccl22*, and *Ccl24*. M2b macrophages are immunity-related, can be induced by lipopolysaccharide (LPS) and have a relatively high expression of *Ccl1*, interleukin 6 (*Il6)*, *Tnfα* and *Il10*. Despite the expression of pro-inflammatory genes, the M2b macrophages protect mice from LPS toxicity [30]. IL10 also mediates the M2c activation with high expression of *Ccl16*, *Ccl18* and, transforming growth factor (*Tgfβ*) [26].

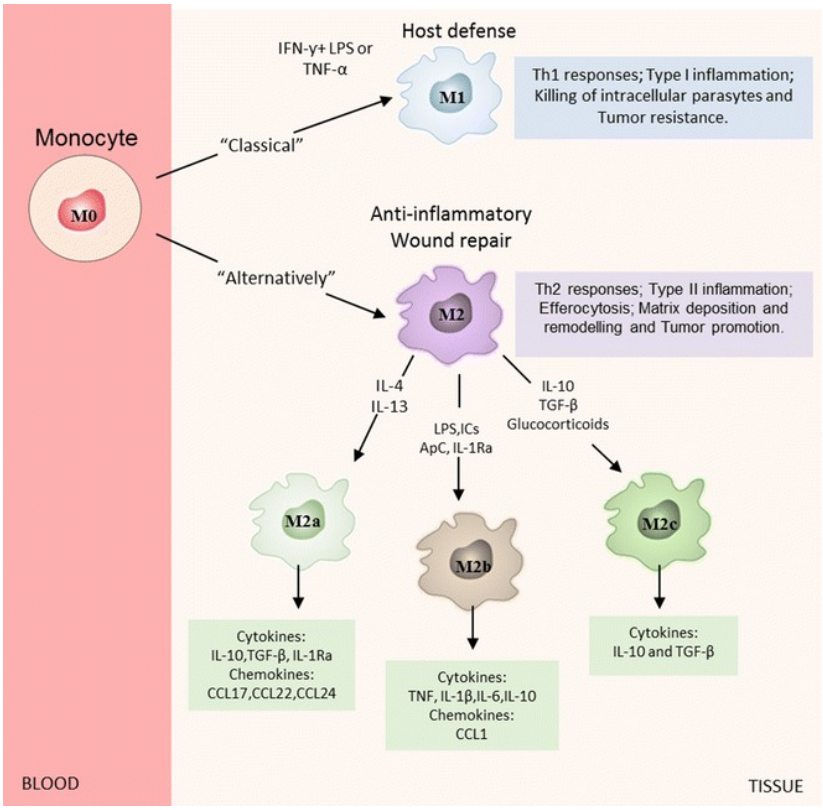

Figure 3. Macrophage activation and polarization.

Different polarized macrophage subtypes are functionally different in response to environment-derived signals. LPS/ IFNγ-induced M1 macrophages are involved in T-cell responses 1 (Th1) responses, type I inflammation, tumor resistance, and clearing pathogens. In contrast, IL4/IL13 medicated M2a macrophages are involved in type II inflammation, allergy, and parasites infection. While M2b macrophages participate in the T-cell responses 2 (Th2) responses and immunoregulation, IL10-stimulated M2c macrophages are mainly involved in inhibiting immune responses and tissue remodeling [31, 32].

1.1.3 Role of macrophages in obesity and T2D

Obesity and its related metabolic disorders such as type 2 diabetes (T2D), liver and cardiovascular diseases are becoming worldwide problems in both developed and developing countries. Longtime sedentary lifestyles and Western diets can be associated with the global epidemic of those obesity-related metabolic diseases [33]. Insulin resistance is the principal mechanism that is involved in the pathogenesis of T2D. Unlike the classical acute inflammation, obesity induces a sustained low-grade chronic inflammation, also referred to metaflammation, which contributes to a series of following pathological changes [34]. In particular, obesity is thought to create a hyperlipidemic 'metabolic stress' microenvironment that induces inflammation in adipocytes. The critical involvement of macrophages in metaflammation is supported by several early clinical studies, which showed a strong correlation between macrophage infiltration in the adipose tissue of obese patients and the disease development [34]. In addition, ablation of macrophages or macrophage-derived key pro-inflammatory cytokines and chemokines significantly restore disease progression in obese mice models [35-38].

Insulin resistance reflects early glucose dysregulation in obesity. With the blunted insulin signaling pathways in the peripheral tissues such as adipose tissues, muscle, and liver, the uptake of glucose into those tissues is halted [39]. As a result, pancreatic β-cells need to produce sufficiently more insulin to antagonize this pathological change, overloaded of the pancreatic β-cells leads to cell death, followed by dropping of insulin secretion which leads to hyperglycemia and T2D [40]. Evidence shows that chronic tissue inflammation can lead to obesity-related insulin resistance. Obesity induces an insulin resistance state in these above tissues through a group of endocrine signaling pathway cross-talk [39].

Adipose tissue not only stores excess energy but also secretes fatty acids and adipokines including leptin, adiponectin, visfatin, vaspin, retinol-binding protein 4 (RBP4) and fibroblast growth factor 21 (FGF21) [41, 42]. Leptin, adiponectin, vaspin, and FGF21 promote while RBP4 impairs insulin sensitivity [43-45]. During the obesity process, the adipose tissue is infiltrated with bone marrow-derived macrophages (BMDMs), which is the primary reason for chronic tissue inflammation [39, 46].

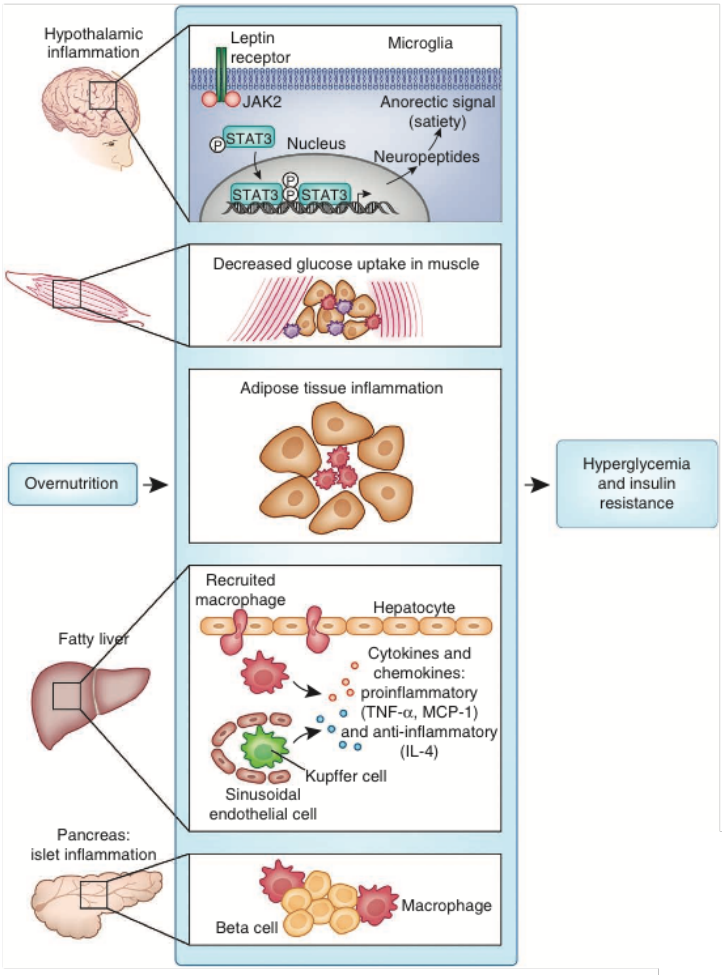

Figure 4. Macrophage infiltration in tissues during obesity.

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Obesity induces macrophage infiltration in different tissues including adipose tissue, muscle, liver, and brain along with increased levels of cytokines and chemokines (**Figure 4**) [47]. The hyper-nutrient microenvironment activates the abundantly recruited resident macrophages in insulin-sensitive tissues such as liver, adipose tissue, and muscle, thereby contributing to the chronic inflammation and metabolic dysregulation. The unique metabolically-activated inflammatory (metaflammatory) macrophage subtypes express cytokines like TNFα, inducible nitric oxide synthase (iNOS) and some chemokines like

CCL2, CCL7 [40, 47].

Interestingly, CCL2 seems to have a particular role in inducing local macrophage proliferation, leading to increased macrophage accumulation characterized as crown-like structures (CLS) in adipose tissues. In contrast to acute inflammation, where macrophages or monocytes are activated by endotoxins (LPS), the hyperlipidemia condition in obesity is also able to activate macrophages via interacting with the TLR2/4 receptors, thereby transcriptionally inducing gene expression of the above pro-inflammatory cytokines and chemokines [48].

1.2 Transcriptional regulation in macrophages

1.2.1 Mechanisms, transcription factors, and co-regulators

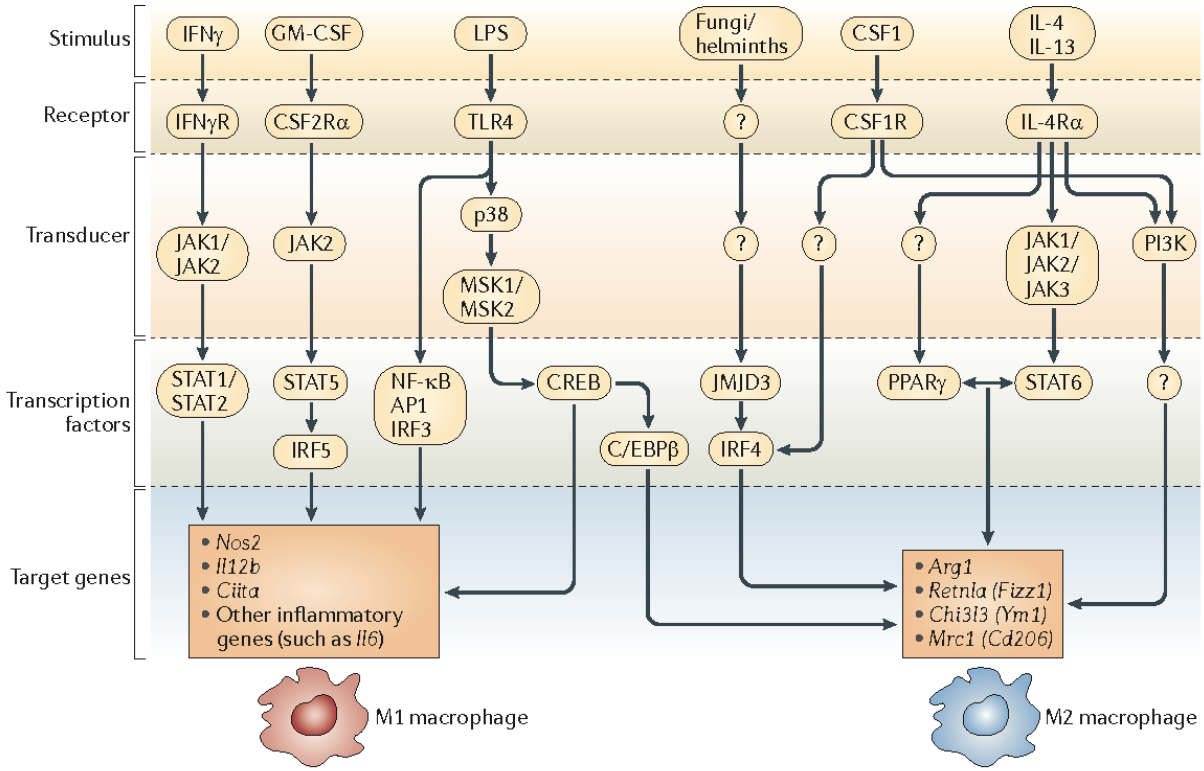

Figure 5. Transcription factor regulation in macrophage polarization.

Reprinted from Nature Reviews Immunology, Vol. 11, p.750-761, Toby Lawrence and Gioacchino Natoli. Transcriptional regulation of macrophage polarization: enabling diversity with identity, doi:10.1038/nri3088, Copyright 2011, with permission from Springer Nature.

Groups of stimulus-responsive TFs (SRTFs) such as nuclear factor kappa B (NF-κB) and lineage-determining TFs (LDTFs) such as PU.1 are involved in macrophage polarization (**Figure 5**) [8, 49, 50]. In IFNγ-induced M1 macrophages, IFNγ binds to IFNγ receptor

(IFNγR) and active signal transducer and activator of transcription 1 (STAT1) and 2 (STAT2), which are the main TFs for M1 polarization [51]. In TLR4-activated M1 macrophages, the ligand (LPS) binds to TLR4 and activate activator protein 1 (AP1) and NF-κB, which are dependent on myeloid differentiation primary response 88 (MYD88). Interferon regulatory factor 3 (IRF3)-medicated *IFN-β* expression is a TRIF-dependent pathway in LPS induced M1 macrophages [52]. CAMP-responsive element-binding protein (CREB), as a ZIP family TF, is involved in M2 polarization. LPS can activate CREB-C/EBPβ pathway to induce M2b macrophage through the immune response. In the M2b process, the M2 classic genes are inhibited by LPS while the LPS-mediated inflammatory genes are unaffected. The M2b-mediated CREB-C/EBPβ pathway is crucial for wound healing [49, 53]. In the IL4/IL13-mediated M2 process, STAT6 is critical for the gene activation [49].

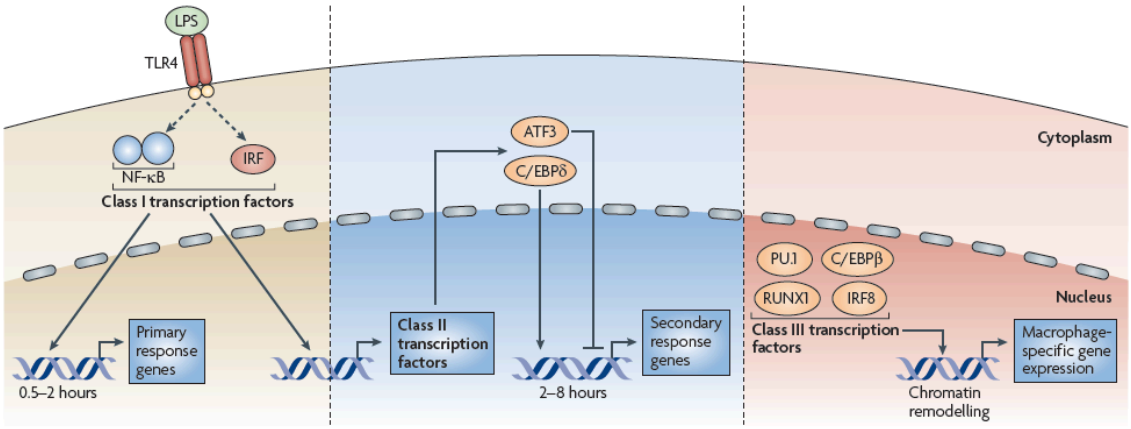

Figure 6. LPS /TLR4 signaling in macrophages.

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Transcriptional regulation in macrophage activation is dynamic. One example is LPS (endotoxin) tolerance which represents a gene-selective LPS resistance status upon a second wave of stimulation after a chronic first wave of LPS treatment. Genes such as *Tnfα* and *Il6* are still responsive to the second wave of LPS stimulation while others such as *Ccl2* and *Ccl7* can no longer be induced by LPS. The physiological function of LPS tolerance is to decrease the excessive inflammatory response and allow the immune system to remain active. The endotoxin tolerance process can be divided into three stages involving different TFs. In the early stage, LPS rapidly induces primary response genes such as *Tnfα* through NF-κB and IRFs. In the second stage, LPS activates TFs such as C/EBPδ. In the third stage, LPS induces a few TFs including PU.1, C/EBPβ, RUNX1, and IRF8, which play roles in

the macrophage differentiation (**Figure 6**) [50]. These three stages are not independent but are functionally integrated within the transcriptional process.

Beyond the key inflammatory TFs (PU.1, NF-κB, AP1, IRFs), several nuclear receptors (LXRs, PPARs), transcriptional co-regulators (co-activators, co-repressors), and kinases including c-Jun N-terminal kinases (JNK) and the IκB kinase (IKK) are involved in M1 macrophage activation [34, 54]. The peroxisome proliferator-activated receptors (PPARs) are a group of metabolism-associated nuclear receptors. There are three isoforms of PPARs, namely PPARα, PPARβ/δ, and PPARγ, which are encoded by distinct genes and which are abundantly expressed in multiple metabolic tissues including macrophages. PPARs are reported to be involved in fatty acid metabolism; however, PPARs can also influence inflammation and immunity in macrophages. Activated PPARs can repress inflammatory genes such as *Ccl2, Il6* in the presence of ligand. PPARs regulate transcription via heterodimerizing with RXRs upon ligand stimulation. Up to now, synthesized ligands targeting PPARα (fibrates) and PPARγ (T2Ds) have been clinically applied for lipid-lowering and insulin-sensitizing purposes [55, 56].

The liver X receptor LXRα is highly expressed in liver, kidney, macrophages, lung, spleen, intestine, brain and adipose tissues, while LXRβ is expressed in almost all tissues and organs. The natural ligands for LXRs are oxysterols, oxygenated cholesterol, and cholestenoic acid. Synthetic agonists such as T0901317 and GW3965 were later developed for pharmacological studies. After ligand binding, LXRs bind to LXR response elements (LXRE), and activate LXR target gene transcription. LXRs are involved in lipid and cholesterol metabolism in macrophages. Activation of LXRs in macrophages leads to the transcription of several genes that are involved in lipid metabolism and reverse cholesterol transport such as *Abca1* and *Abcg1* [57-59].

Depletion of nuclear receptor co-repressor 1(NCOR) in macrophages is associated with LXR activation linked with altered omega-3(ω-3) fatty acid metabolism and improved metabolic phenotypes in a mouse model of diet-induced obesity [60]. Unlike PPARγ, which is rapidly downregulated upon inflammatory stimuli (e.g. LPS, unpublished data), both LXRα and β remain expressed during inflammation, potentially indicating LXRs as more potent drug targets for therapeutic intervention. LXRα is additionally involved in defining splenic macrophage specification [61]. LXR activation was reported to repress LPS-

induced inflammatory gene transcription via trans-repression, i.e. through inhibition of inflammatory TFs such as NF-kB [62, 63].

Last but not least, so-called co-regulators are crucial components of transcriptional regulation in macrophages. Co-regulators interact with and modulate the activity of TFs, and they function within larger multiprotein complexes to modify chromatin states linked to transcriptional activity. Inflammatory gene expression requires co-activator complexes possessing histone acetylase (HAT) activities, formed by CREB-binding protein (CBP) and the related E1A binding protein p300 (p300) to remodel nucleosome activity [64, 65]. In contrast, multiple co-repressor complexes, including those possessing histone de-acetylase (HDAC) activities, are required to maintain repressed states and to silence gene expression upon activation (feedback control) in macrophages [65, 66].

1.2.2 The GPS2 subunit of the HDAC3 co-repressor complex

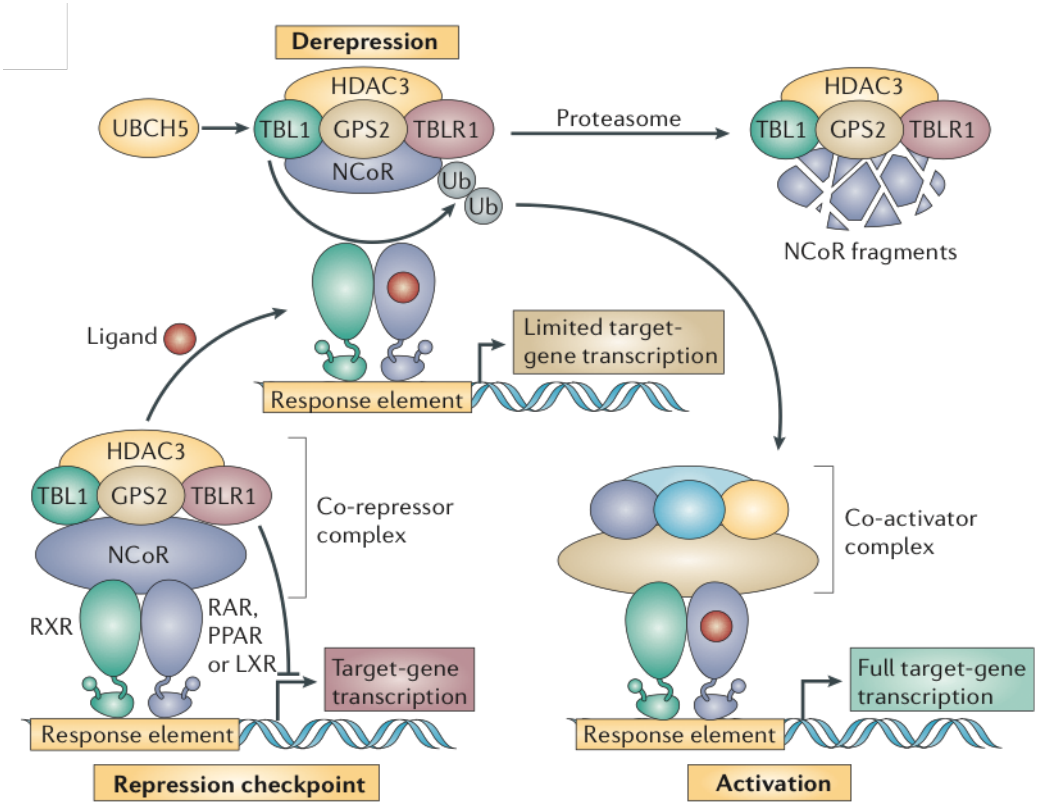

Figure 7. HDAC3 co-repressor complex in control of inflammatory gene transcription.

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Co-repressor complexes formed by NCOR and the related silencing mediator of retinoic-acid and thyroid-hormone receptor (SMRT) were discovered in 2000 by different groups [67-69]. The NCOR/SMRT complex contains transducin b-like 1 (TBL1), TBL1-related

protein (TBLR1), G-protein pathway suppressor 2 (GPS2) and histone deacetylase 3 (HDAC3). These core subunits interact with each other and assemble into a main co-repressor complex to inhibit gene transcription in macrophages and other cell types. When cells are exposed to a particular stimulus such as pro-inflammatory cytokines, the HDAC3 complex releases, allowing co-activators to bind and to stimulate transcription (**Figure 7**) [65].

NCOR (also known as N-CoR, NCOR1) is a corepressor of a broad range of TFs. It is a large protein of approximately 270 kDa and it is expressed seemingly ubiquitously, i.e. in all tissues. NCOR contains two distinct C-terminal domains that are required for interactions with nuclear receptors [70], while interactions with multiple TFs require other domains. SMRT (also known as NCOR2, nuclear receptor co-repressor 2) is a NCOR related factor that was discovered in 1995 [71]. SMRT and NCOR share similar structures and have conserved functional domains, which allows them acting in part redundant.

Histone deacetylases (HDACs) are a group of proteins that regulate histone and non-histone protein acetylation and deacetylation [72]. The HDAC superfamily is classified into four classes depending on sequence homology. HDAC1, HDAC2, HDAC3, and HDAC8 belong to class I. HDAC3 is ubiquitously expressed and the major enzymatic component of the NCOR/SMRT co-repressor complex [73].

GPS2 (also named AMF1) was initially identified in genetic screens for suppressors of G-protein mutations in *Saccharomyces cerevisiae* [74]. GPS2 is a highly conserved 37 kDa protein, containing 327 amino acid residues in mice and humans, which is expressed in most tissues. GPS2 was also found to interact with virus proteins such as human papillomavirus (HPV), bovine papillomavirus type 1 (BPV-1) and Hepatitis C virus (HCV) [75, 76]. The transcriptional functions of GPS2 started to become evident when it was subsequently identified as a subunit of the HDAC3 co-repressor complex [77]. Interestingly, GPS2 could inhibit TNFα-induced JNK activation, thus discovering the first link to inflammatory pathways [78]. Other studies revealed that GPS2 also can influence c-Jun, p53, and p300 activities and thereby regulate the transcription of inflammatory and cancer-related genes [79, 80]. Studies by the Treuter laboratory identified GPS2 as a significant regulator in bile acid biosynthesis: In human hepatocyte cell lines, GPS2 regulates cytochrome P450 7A1 (*CYP7A1*) and cytochrome P450 8B1(*CYP8B1*) expression which are the two major enzymes in bile acid pathway [81]. Later on, Jakobsson et al.

reported that in human hepatocytes and macrophages GPS2 was required for the LXR-dependent expression of *ABCG1*, a major cholesterol transporter in macrophages [82]. Zhang et al. also showed that GPS2 interacts with Regulatory Factor X4 Variant 3 (RFX4v3) and trans-activates CX3C-type chemokine gene expression [83]. These few studies indicated that GPS2 does in certain contexts also work as a 'co-activator' in macrophages.

There is growing evidence that altered expression and function of subunits of the HDAC3 co-repressor complex associates with certain cancers and metabolic diseases. Research uncovered the reduced expression of NCOR in breast and bladder cancers cell lines [84, 85] and *in vivo* experiments showed NCOR function as an oncogene through transcriptional regulation [86]. GPS2 expression was down-regulated in liposarcoma, and depletion of GPS2 *in vitro* in a liposarcoma cell line increased proliferation and migration [87], suggesting GPS2 to act as a tumor suppressor in liposarcoma. TBL1 was reported as a tumor suppressor gene in pancreatic cancer cells by controlling cell proliferation and invasion [88]. TBL1 was further implicated in controlling liver steatosis in metabolic syndrome patients [89]. The related TBLR1 acts as a co-activator to repress prostate cancer proliferation via the androgen receptor (AR) [90]. Jiao et al. reported that HDAC3 was upregulated in human pancreatic cancer and depletion of HDAC3 decreased the cell proliferation *in vitro* [91].

Low-grade chronic inflammation can cause adipose tissue dysfunction and is a major characteristic and risk factor of obesity. Toubal et al. reported that GPS2 and SMRT were significantly reduced in adipose tissue of obese humans, as compared to non-obese, and this was inversely correlated to the state of metaflammation (i.e. macrophage infiltration in the adipose tissue, systemic inflammation, insulin resistance). After gastric bypass surgery, the patients´ inflammatory status was improved along with a restored expression of GPS2 and SMRT, suggesting a causal relationship [92]. The key inflammatory mediators in adipose tissue, such as CCL2, IL6, and IL8, and the regulation by the NCOR co-repressor complex are considered to be particularly critical for obesity development. These findings point at a particular function of the HDAC3 co-repressor complex in obesity and suggest that alterations of epigenetic regulatory networks potentially underly the chronic inflammatory status of obese adipose tissue [92].

1.2.3 Knockout mouse models for HDAC3 co-repressor complex subunits

Although the co-repressor complex subunits are assumed to physiologically function together, the reported knockout mouse models for NCOR, HDAC3 and TBLR1 display different phenotypes (**Table 1**). Adipocyte-specific TBLR1 knockout mice result in obesity and metabolic dysfunction through reduced lipolysis [93]. Surprisingly, NCOR knockout mice in both adipocytes and macrophages display anti-inflammatory phenotypes [60, 94]. High-fat diet (HFD) induced insulin sensitivity, improved glucose levels, and reduced inflammation. The NCOR knockout phenotype in adipocytes may be due to up-regulated PPARγ target genes and enhanced insulin sensitivity by increased cyclin-dependent kinase 5 (CDK5)-mediated PPARγ phosphorylation [94]. In contrast, the NCOR knockout phenotype in macrophages may be due to elevated LXR-dependent ω3-fatty acids gene expression, which subsequently caused inflammatory gene repression [60].

Table 1 Knockout mouse models for subunits of the HDAC3 co-repressor complex.

Protein	Mouse model	Phenotypes	High-fat diet (HFD)	Mechanism
SMRT	Macrophage knockout	-	-	-
	Adipocyte knockout	-	-	-
NCOR	Macrophage knockout	Anti-inflammatory	Reduced inflammation, improve glucose tolerance, enhance insulin sensitivity	De-repression of LXR-mediated ω3-fatty acid synthesis [60]
	Adipocyte knockout	Anti-inflammatory	Increased obesity, improve glucose tolerance, enhance insulin sensitivity	Changes in PPARγ phosphorylation [94]
HDAC3	Macrophage knockout	Anti-inflammatory	-	Epigenetic modifications such as histone acetylation activity changes [95, 96]
	Adipocyte knockout	Lethal [97]	-	-
	Adipocyte knockout	Enhanced browning of white adipose tissue	No effect	Activation of *de novo* fatty acid synthesis and β-oxidation [98]
TBLR1	Macrophage knockout	-	-	-
	Adipocyte knockout	Pro-inflammatory [93]	Aggravated adiposity, insulin resistance, glucose intolerance	-

Macrophages were unable to induce many inflammatory genes expression such as *Il6*, *Ptgs2*, *Tnfα* upon knockout of HDAC3 [95], which increased *Cox1* expression and inhibited basal and LPS-induced *Ifnb1* gene expression that could impair the secondary response to LPS. On the other hand, it was reported that HDAC3 functions as an epigenomic brake in alternative M2 macrophage activation. When exposed to IL4/IL13, HDAC3-deficient macrophages had elevated M2 marker gene expression [96]. HDAC3 knockout in white adipose tissue was lethal by using fatty acid binding protein 4 (*Fabp4*)-Cre mice, which the indicates the crucial roles of HDAC3 in adipose physiology [97]. However, another group reported the HDAC3 knockout using adiponectin-Cre mice regulated the white adipose tissue metabolism and function [98].

1.3 Enhancers - key elements of the macrophage epigenome

1.3.1 Enhancer structure and function relationship

The term 'enhancer' was first coined in studies using simian virus 40 (SV40) over 30 years ago [99]. Schaffner described that a 72-bp SV40 element could increase the expression of the rabbit β-globin gene in HeLa cells and further studies showed that this element could enhance transcription in many other promoter contexts [99, 100]. Since then, an enhancer is defined as a cis-regulatory DNA element which can independently activate the downstream gene transcription from a distance. Such regulatory elements contain specific recognition sequences for multiple TFs that enables precise spatiotemporal patterns of gene expression during development and cellular response to stimuli. Enhancers positively regulate the transcription activity of promoters and are thus critical for gene expression. Deleting enhancers can affect histone modifications and loop-mediated chromatin structures [101, 102].

High-throughput DNA sequencing techniques such as ChIP-sequencing (ChIP-seq) and DNase I hypersensitive sites sequencing (DNase-seq) enabled researchers to discover enhancers in a genome-wide scale [103-106]. The histone modifications H3K27ac and H3K4me1 are usually used as enhancer markers and some LDTFs such as sex determining region Y-box 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) and mediator (e.g. MED1) were also reported as enhancer markers in specific cell types [101, 107, 108]. Thus, the recruitment of these factors along with specific histone markers can be used to map and evaluate enhancer activities.

The general regulatory functions of enhancers are mediated through several mechanisms. 1) Recruiting and releasing of enhancer-regulating TFs. For example, estrogen receptors (ERs), forkhead box protein A1 (FOXA1), LXR, and PU.1 bind to enhancers and promoters are dependent on the DNA-binding motifs [82, 109-111], which causes the transcription of target genes. 2) Transcriptional output via enhancer-derived non-coding RNAs (eRNAs). The transcription on the enhancer itself can affect the enhancer and promoter communication. There is evidence suggesting that eRNAs are functionally required for gene transcription and not just transcription noise or non-functional byproducts of enhancer transcription [112-114]. 3) Maintenance of enhancer-promoter looping structures. enhancer-promoter loops are another critical mechanism for gene inter-regulation. Genome-wide enhancer-promoter interactions (by Hi-C) and individual genes loop formation (by 3C and modified 4C, 5C) are primarily studied [115-117], showing that loop formations are common events in nuclear chromatin/DNA organization.

Compare to typical enhancers, super-enhancers tend to span larger genomic regions of high chromatin accessibility, which enrich RNA polymerase II (Pol II), MED1, bromodomain containing 4 (BRD4), eRNAs, p300, CCCTC-Binding Factor (CTCF)/cohesin, H3K27ac, H3K4me2, and H3K4me1 [118]. Super-enhancers are cell type-specific and often found in the genes which have cell type-specific functions. In cancer cells, super-enhancers are enriched at oncogenes such as MYC proto-oncogene (MYC) [108, 119, 120]. In embryonic development, super-enhancers are assembled by the stem cell TFs such as Oct4, Sox2, kruppel-like factor 4 (Klf4), and homeobox transcription factor nanog (Nanog) [107].

The well-explained mechanisms for the regulation of enhancers on target genes are enhancer-promoter communication, which requires the formation of 3D chromatin looping structures [116]. Generally, the enhancer-promoter loop formation is required by the enhancer, cohesin-loading factor, DNA binding proteins, and other transcription machinery proteins such as Pol II and promoter. Nowadays, the loop-mediated gene regulation is commonly accepted while the emerging evidence shows the eRNAs are crucial for loop formation and gene expression [121].

Macrophage activation is also largely dependent on enhancers. According to mouse studies, there are about 1800 macrophage-specific genes along with 4700 macrophage-specific enhancers [122]. In response to stimuli such as LPS and IFNγ, macrophage gene activation

is profoundly marked by histone H3K4me1, H3K4me2, low enrichment by H3K4me3, and are controlled by the LDTF PU.1, C/EBP, AP1 [101, 111]. Notably, in both M1 and M2 activation, macrophage *de novo* enhancers (latent enhancers) play a central role in gene expression. In M1 activation, some pre-existing enhancers marked by H3K4me1, H3K4me2, H3K27ac, and PU.1 can loose, and others can gain function [111, 123]. In M2 activation, about 260 active enhancers control 314 responsive genes [122]. Pol II transcription which overlapped with the histone markers are principally enriched in macrophage activation, and global analysis shows the enhancer activities are correlated with eRNA transcription linked with nearby gene expression [124].

Functional genomic studies have shown that super-enhancers are required for gene expression, cell identities, tumor pathogenesis, and cell development. For example, super-enhancers can be used as biomarkers for tumor-specific pathologies [119]. Graham et al. also showed that the nuclear receptor subfamily 4 group A member 1 *(Nr4a1)* enhancer deletion could affect the development of Ly6Clow monocytes with minor effects on inflammation [125]. Krüppel-like factor 2 (KLF2) specifically binds to the target enhancer regions and controls monocyte differentiation [125]. Another study has demonstrated that deletion of a *MYC* oncogene core enhancer in mice resulted in resistance to intestinal tumorigenesis while not leading to global *MYC* inactivation, indicating cell specificity of enhancers [126]. Moreover, Xiao et al. demonstrated super-enhancer medicated *Il9* expression in T helper type 9 (Th9) cells in airway inflammation, which provides therapeutic potential for clinical applications [127].

Overall, while there are more than 150,000 enhancers in human and mouse cells, only a few enhancers have been functionally tested so far. Therefore, our current understanding of enhancer function in mammalian cells, including macrophages, is limited. For example, it is currently very difficult to predict how many functional enhancers control a given gene, how many genes are controlled by a given enhancer, and how diverse inflammatory signals control individual enhancers and linked genes.

1.3.2 Role of enhancer-transcribed eRNAs

While only 1% of the human genome consists of protein-coding genes, numerous studies have recently uncovered that more than 60% is transcribed into diverse non-coding RNAs [105]. eRNAs are non-coding transcripts that originate from H3K27ac-, H3K4me2- and

H3K4me1-marked enhancer regions. eRNAs are less than 2000 bp in length and usually do not have splice variants. Consistent with their origin, the expression of many eRNAs is highly tissue-specific and poorly conserved between species. Non-polyadenylated eRNAs are much more frequent than polyadenylated eRNAs [128-130]. Caused by the lack of polyadenylation and termination sites, eRNAs can be easily degraded by exosomes [129].

Enhancer transcription involves two main steps, i.e. the enhancer priming, and enhancer transcription initiation. In the first step, pioneer TFs, LDTFs, and co-regulators cooperate to open chromatin and modulate histones at enhancers. In the second step, additional key TFs including SRTFs, CBP/p300, MED1, and Pol II are recruited to initiate transcription [128]. As with messenger RNA (mRNA) transcription, eRNAs may be additionally controlled at the level of elongation.

Due to the low abundance and easy-to-degrade characteristics of eRNAs, detection of eRNA remained technically challenging. In addition, as most of eRNAs lack polyA, the polyA-based RNA-sequencing (RNA-seq) is not suitable for detecting eRNAs. Reverse transcription-PCR (RT-PCR) can be used for the eRNA detection when using random primers [131]. Global run-on sequencing (GRO-seq) and its improved versions is a robust method to identify eRNAs [113, 132, 133]. RNA fluorescence *in situ* hybridization (RNA-FISH) is a DNA probe-based method to ascertain the localization of individual eRNAs [134, 135]. Pol II ChIP-seq provides indirect evidence to show the correlation between the target genomic region and the transcription products [124, 131].

How exactly eRNAs regulate transcription is not yet understood. However, eRNAs share common features with other types of long non-coding RNAs (lncRNAs) which may help to propose mechanisms of eRNA function (**Figure 8**) [130]. There are currently at least three models to explain the transcriptional regulatory mechanisms of lncRNAs. The first model is that the lncRNA can recruit the transcription machinery to target gene promoters. For example, the steroid receptor RNA activator (SRA), HOXA transcript at the distal tip (HOTTIP), Miranda (Mira) and distal-less homeobox 6 antisense RNA 1(DLX6-AS1) were reported to recruit TFs and activators to enhance transcription [136-139]. The second model is that lncRNAs facilitate enhancer-promoter loop formation [140]. The third model is that lncRNAs such as *Braveheart* [141] can activate target gene expression by promoting the release of co-repressors.

A few studies suggest that lnc/eRNAs play crucial roles in macrophage differentiation and stimulus-response [142]. The lincR-Ccr2-5′AS expression can regulate the Th2 related inflammatory gene expression. The ncRNA THRIL recruits ribonucleoprotein L (RNPL) to enhancers to control TNFα expression [143]. Xiao et al. reported that the IL9 super-enhancer and its related eRNA control airway inflammation [127].

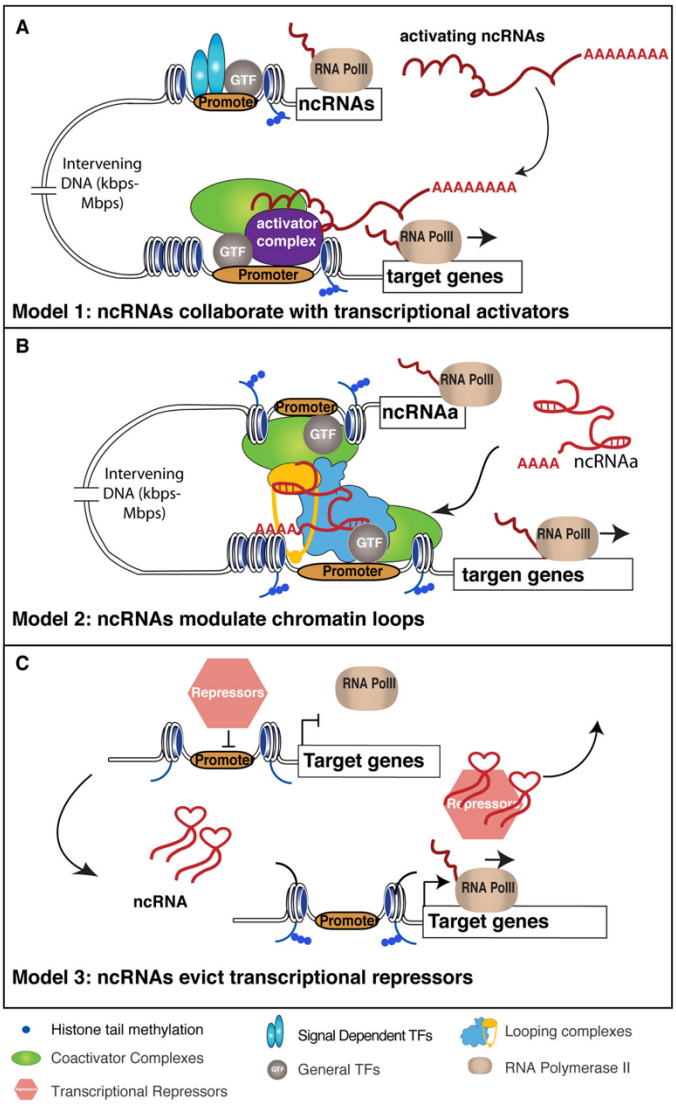

Figure 8. Models of lncRNA function that may apply to eRNAs.

Reprinted from Trends in Biochemical Science, Vol 39. p.170-182, Michael T.Y. Lam et al., Enhancer RNAs and regulated transcriptional programs. doi: 10.1016/j.tibs.2014.02.007, Copyright 2014, with permission from Elsevier.

Despite numerous studies which support the functionality of eRNAs, the relevance and implications are currently debated and there is no final consensus as to the mechanisms of eRNA regulation and function. One challenging part is that eRNAs are expressed at low levels and unstable, and most of them are expressed only in the nucleus, which make approaches to degrade eRNAs via RNA interference (RNAi) not suitable [144-146]. Using a polyadenylation signal to terminate eRNA expression will perhaps provide an alternative

way to interfere with eRNA function [147, 148]. Related inhibitory experiments that specifically target the eRNA and not the process of enhancer transcription would be needed to test for function. Until then, the key argument that enhancer transcription is non-functional ‘noise’, i.e. a byproduct of Pol II at open enhancer chromatin, cannot be dismissed [121, 149].

2 OBJECTIVES AND AIMS

2.1 General Objective

The objective of this thesis is to characterize the functions of the anti-inflammatory co-regulator protein GPS2, and of GPS2-containing multi-protein complexes, at the molecular and physiological level. Particular emphasis will be on dissecting transcriptional pathways and epigenome alterations in macrophages that potentially link GPS2 to metabolic-inflammatory diseases.

2.2 Specific Aims

1) To determine the role of GPS2 in RCT in macrophages. Focus on characterizing metabolic and inflammatory signals, key target genes and key TFs as well as the role of other corepressor complex subunits.

2) To investigate how the GPS2-containing co-repressor complex regulates pro-inflammatory macrophage enhancers linked to genes of significance for the development of obesity-linked T2D. Focus on enhancers and eRNAs potentially controlling the expression of the chemokine *Ccl2*, a major GPS2 target in adipose tissue-macrophages.

3) To generate and characterize macrophage-specific *Gps2* knockout mice and cell lines. Focus on integrative physiology and epigenomics, i.e. studying the causal relationship between loss-of-GPS2, epigenome alterations and phenotypes potentially linked to metaflammatory diseases.

4) To characterize liver-specific *Gps2* knockout mice. Focus on the consequences of GPS2 depletion on lipid metabolism and on the development of obesity-induced non-alcoholic fatty liver disease (NAFLD).

3 METHODOLOGICAL CONSIDERATIONS

A detailed description of all experimental and computational methods used in the thesis are provided in the individual publications or manuscripts. The goal of this section is to discuss the advantages and disadvantages of key methods.

3.1 Mouse models and primary macrophages

In all studies, we used macrophage-specific *Gps2* knockout mice, along with wild-type (floxed) mice, in the C57B1/6J background to investigate GPS2 functions in inflammation and metabolism [150]. We isolated primary mouse bone marrow-derived macrophages (BMDMs) and mouse thioglycollate-elicited peritoneal macrophages (TEPMs) and used LPS to induce macrophage M1 inflammation response [150-152].

3.2 Macrophage cell lines

The main macrophage cell line used in this study was RAW 264.7, which was purchased from the American Type Culture Collection (ATCC, TIB-71). The human macrophage cell line THP1 (ATCC, TIB-202) was additionally used in **Paper I**. Both RAW264.7 and THP1 cells are important and widely used *in vitro* tools for immunology research. RAW264.7 is a macrophage-like cell line, which originated from a leukemia mouse in 1978. Compared to primary mouse-derived BMDMs and TEPMs, the RAW264.7 cell line has similar genomic structure, gene expression patterns and immune responses and can be easily cultured *in vitro*, use serving a relevant alternative *in vitro* model in line with the 3R-principle.

3.3 CRISPR/Cas9-based gene editing

The clustered regularly interspaced short palindromic repeats (CRISPR) gene-editing technology was used in this thesis to delete non-coding (e.g. enhancers, promoters) and coding (e.g. *Gps2*) DNA sequences in RAW 264.7 cells. CRISPR and its related genes are essential for adaptive immunity in bacteria, enabling the organisms to eliminate invading genetic changes [153]. We further used modifications referred to as CRISPRi/dCas9 to disturb transcription without DNA editing [154-156].

3.4 Adenovirus and lentivirus-mediated RNAi knockdown

In order to silence gene expression via knockdown of a particular mRNA *in vitro*, RNAi using short hairpin RNAs (shRNA) is used in this thesis. Basically, shRNAs are artificial RNAs that have a hairpin turn, which is degraded in a process catalyzed by the Dicer enzyme. This process will cause the RNA-induced silencing complex (RISC) to bind to the target mRNA and cause subsequent degradation. Both adenovirus and lentivirus were used to deliver the shRNAs. Because macrophages have low transfection rates using conventional small interfering RNAs (siRNA), the virus-based shRNAs provide potent alternatives to silence gene expression in macrophages. Through the **Paper I** and **Paper II** we mainly used lentivirus to knockdown particular genes via generating stable cell lines.

3.5 Chromatin immunoprecipitation-coupled sequencing (ChIP-seq)

ChIP-seq is a powerful tool to investigate the genome-wide binding profile of a particular TF, co-regulator or epigenetic histone mark such as H3K27ac and H3K4me3. Despite there are many in part distinct experimental procedures, high-quality ChIP-seq data are largely dependent on the quality of the antibody and the crosslinking and sonication conditions which can differ amongst cell-types and targets. Antibody quality is the most critical issue for ChIP-seq. In this thesis, we performed ChIP-seq for GPS2 (using self-made custom rabbit polyclonal antibodies raised against N- and C-terminal GPS2 epitopes), H3K27ac and H3K4me3 in RAW264.7 cells.

3.6 RNA sequencing (RNA-seq, GRO-seq)

Next-generation sequencing (NGS) technologies have revolutionized the genome-wide analysis of transcriptomes via RNA-seq. RNA-seq identifies globally differential expression of both known and unknown genes and non-coding RNAs, which cannot be detected using probe-based microarray methods. RNA-seq is usually a Pol II-based method, which will involve a poly(A)-mRNA selection step [157]. In this thesis, we used Illumina HiSeq 2000 for global transcriptome sequencing.

Global run-on sequencing (GRO-seq) is another high-throughput sequencing method which detects nascent RNAs, both coding pre-mRNAs and different classes of ncRNAs including

eRNAs [111, 158]. In this thesis, we have established and applied GRO-seq in RAW264.7 cells and BMDMs (in collaboration with Dr. Minna Kaikkonen, Kuopio University).

3.7 Cholesterol efflux assay

Macrophages play a critical role in RCT by transporting intra-cellular cholesterol to plasma in the form of HDL. ABCA1 and ABCG1 are the major macrophage cholesterol transporters in mice and humans, the expression of which is regulated by oxysterols (via LXRs) and inflammatory signals (likely via inflammatory TFs, to be characterized). The cholesterol efflux assay is used to test the capacity of cells to export cholesterol. In this thesis, we used a fluorescence-based cholesterol efflux assay to examine the influences of GPS2 on cholesterol transportation. Comparing to the typical [^{3}H] cholesterol based method; the fluorescence method is non-radioactive and high-throughput.

3.8 Locked Nucleic Acids (LNA)-based eRNA depletion

Although small interfering RNAs (siRNA) are widely used to degrade mRNAs via RNase A in the cytoplasm, they cannot be used to degrade nuclear-localized ncRNAs including eRNAs. This can be achieved via antisense Locked Nucleic Acids (LNA), which are modified RNAs, both *in vitro* and *in vivo.* In this thesis, we used antisense LNAs (obtained from Exiqon/Qiagen) to degrade different *Ccl2* enhancer eRNAs.

4 RESULTS AND DISCUSSION

4.1 Paper I: GPS2 is required for LPS-induced cholesterol efflux

To investigate GPS2 role in macrophages metabolism, we used both CRISPR/Cas9 GPS2 knockout and lentivirus GPS2 knockdown systems in RAW264.7 cells. We observed that GPS2 depletion caused significant down-regulation of *Abca1*, a main cholesterol transporter gene in macrophages, along with up-regulation of chemoattractant genes *Ccl2* and *Ccl7*, upon LPS treatment. Further experiments revealed that GPS2 depletion significantly decreased LPS-mediated cholesterol efflux. Unlike the classic ABCA1 induction by LXR agonists, induction by LPS involved the TLR4/p65 signaling pathway. GPS2 depletion had only minor effects on LXR agonist-induced Abca1 expression, which suggests GPS2 to act independently of LXR. However, GPS2 depletion resulted in the loss of LXR trans-repression, as shown for *Ccl7*. Double knockdown of GPS2 and p65 experiments showed that the expression of *Abca1* was not further inhibited by depletion of p65 in GPS2-depleted RAW cells. These data suggest that GPS2 cooperates with p65 but not with LXRs to induce *Abca1* expression in response to LPS.

While GPS2 mostly acts as a subunit of the HDAC3 co-repressor complex to repress target gene expression, our data further suggest that the activation of ABCA1 was independent of NCOR and SMRT, the major GPS2-binding subunits in the complex. However, HDAC3 knockdown also abolished LPS-induced *Abca1* expression, which may indicate a potential cooperation between GPS2 and HDAC3.

We further dissected the genomic mechanisms behind the GPS2 regulation of *Abca1* expression using ChIP-seq. We compared the GPS2, LXR and p65 ChIP-seq data (i.e. the cistromes of these factors) at the *Abca1* gene locus, which revealed co-occupancy of GPS2 and p65 at *Abca1* gene promoter and enhancers. In contrast, LXR binding was in part different from GPS2, consistent with the LXR-independence of the LPS/GPS2-regulated *Abca1* expression. ChIP experiments at *Abca1* promoter and enhancer further showed that depletion of GPS2 significantly decreased p65 binding upon LPS treatment, and depletion of p65 abolished GPS2 binding, which supports the physical interaction and dependence of the two factors (**Figure 9**).

We finally attempted to verify the key results in the human monocyte THP1 cell line. The data showed that knockdown of GPS2 also decreased LPS-induced *ABCA1* expression while having a minor effect on LXR ligand-induced *ABCA1*expression. This result suggests that the ABCA1 gene regulation by GPS2 and LPS is conserved between humans and mice.

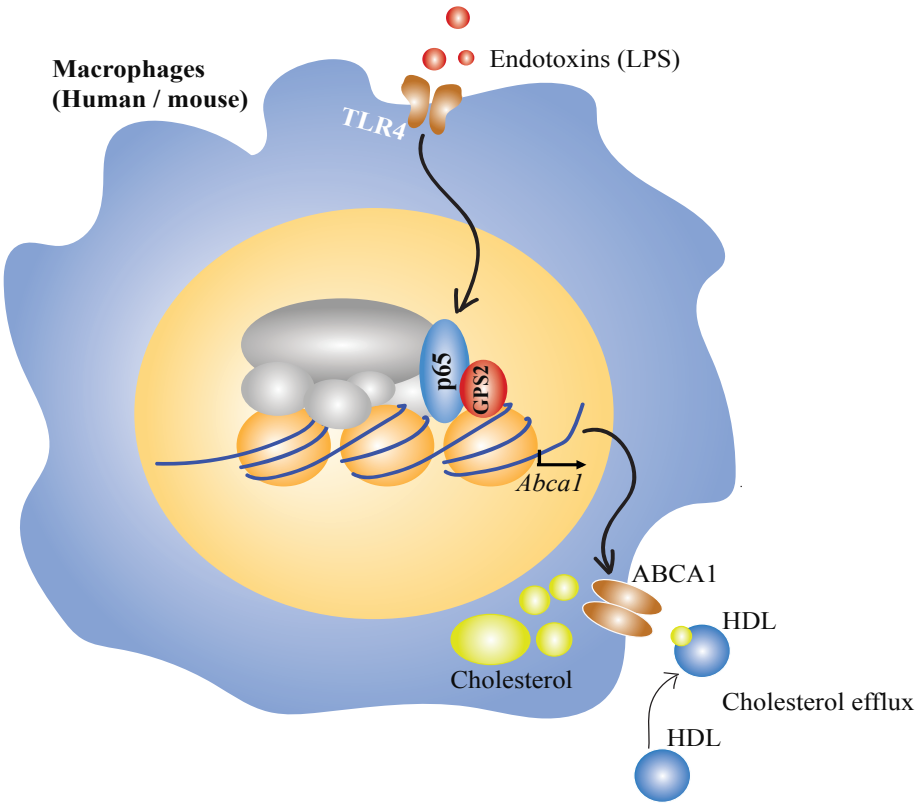

Figure 9. Regulation of ABCA1-dependent cholesterol efflux by GPS2.

Reprinted from The FASEB Journal, Zhiqiang Huang et al., G protein pathway suppressor 2 (GPS2) links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages. https://doi.org/10.1096/fj.201801123R, Copyright 2018.

4.2 Paper II: GPS2 represses macrophage enhancers and eRNA transcription

In order to generate a cistrome profile of GPS2 binding in an *in vitro* macrophage system, we performed GPS2 ChIP-seq in the RAW264.7 cell line upon LPS treatment. Combining GPS2 depletion with RNA-seq transcriptome data, we found GPS2´s binding was enriched at promoters and enhancers to repress expression of inflammatory genes, including *Ccl2* and *Ccl7*. Upon removing GPS2, *Ccl2* expression was upregulated along with a significant enrichment of the histone modification H3K27ac (an active histone/epigenome mark) at both *Ccl2* promoter and enhancers. Kinetic binding analysis upon LPS treatment revealed GPS2 release and re-binding selectively at enhancers of a specific gene cluster (group 2). Notably, group 2 contains many of the top GPS2-repressed target genes. enriched in inflammatory chemokine signaling pathways according to Kyoto encyclopedia of genes and

genomes (KEGG) pathway analysis. Overall, these data revealed a correlation between GPS2 cistrome, epigenome, and transcriptome.

To gain a better understanding of the detailed regulation of GPS2 on its target genes, we choose the most significant gene *Ccl2* to investigate the underlying mechanisms. We compared the ChIP-seq data of the epigenetic histone marks H3K4me3 and H3K27ac with the GPS2 cistrome and found co-occupancy at several *Ccl2* enhancers. To identify which of the GPS2-positive enhancers were functionally required for *Ccl2* expression, we deleted the distal and proximal enhancers using CRISPR/Cas9. We found the distal enhancer E1 was crucial for *Ccl2* expression, while the proximal enhancer E2 was dispensable, despite being marked by GPS2 and H3K27ac. GRO-seq data revealed that upon LPS treatment the enhancer E1 region produced eRNAs, which was repressed by GPS2. We further tested eRNA function of enhancer E1 using LNAs and CRISPR interference, which additionally revealed that eRNA transcription was not affected by promoter deletion.

In sum, these data support the involvement of enhancer E1 eRNA transcription in controlling the LPS-inducible expression of *Ccl2* and the adjacent co-regulated *Ccl7* gene. They point further at a sequence of events during LPS-activation, starting with the release of the GPS2 complex (de-repression), eRNA synthesis, H3K27 acetylation, and mRNA synthesis. While *Ccl2* mRNA synthesis depends on the presence of the *Ccl2* promoter, synthesis of the *Ccl2* eRNAs does not, indicating independent regulation of eRNA transcription. The conclusions of Paper III are summarized in a model (**Figure 10**).

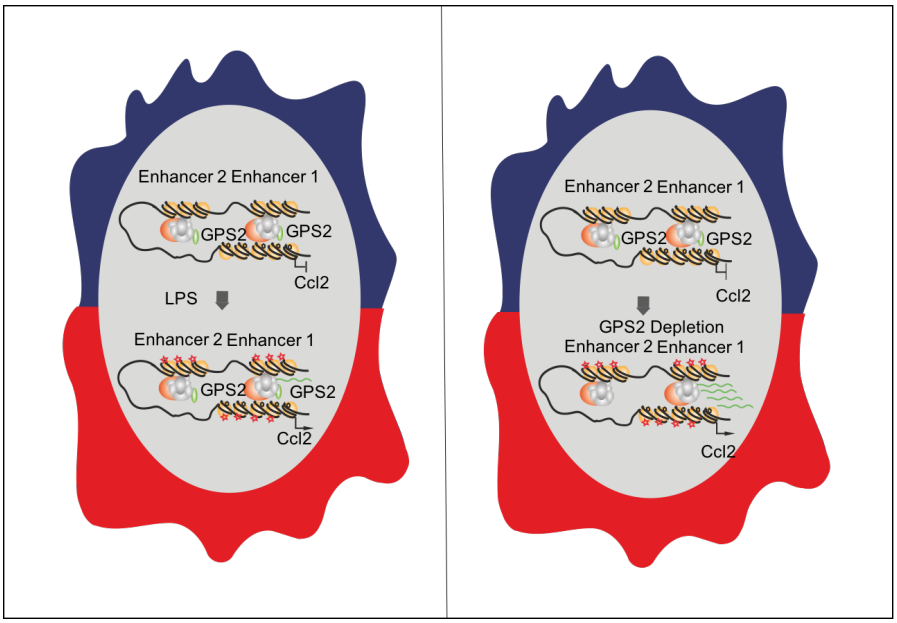

Figure 10. Model of *Ccl2* enhancer repression by GPS2.

4.3 Paper III: Loss of GPS2 triggers metaflammation in mice and human macrophages

The correlation between GPS2, inflammation and T2D risk in human macrophages.

In collaboration with Dr. Nicolas Venteclef (INSERM Paris), we investigated the expression of the genes encoding GPS2 and all other corepressor complex core subunits in obese/T2D human subjects from three distinct populations. In obese patients, *GPS2* expression in adipose tissue macrophages (ATMs) was negatively correlated with inflammatory status and T2D risk score. GPS2 overexpression in ATMs caused the repression of pro-inflammatory chemokine (*CCL2*) and cytokines including interleukin 1 beta (*IL1β)*, *IL6*, *IL8*, and *TNFα*) genes. On the contrary, RNAi-mediated GPS2 depletion in ATMs upregulated theses inflammatory genes. Microarray data also showed that GPS2 depletion in human monocyte-derived macrophages (HMDMs) displayed a pro-inflammatory gene signature. These data suggested that GPS2 in human macrophages, particular in ATMs, could be causally related to inflammation and T2D.

GPS2-deficient mouse macrophages display a pro-inflammatory gene signature and enhanced TLR activation upon infectious or metabolic signaling.

Macrophage-specific GPS2 knockout (MKO) mice were generated and used to investigate GPS2-regulated transcriptomes in BMDMs and TEPMs from MKO versus WT mice using microarrays. We found at the genome-wide scale that the pro-inflammatory GPS2 gene signature, obtained from unstimulated BMDMs, substantially overlaps with the LPS/TLR4 signature obtained from TEPMs. The microarray results additionally suggested that GPS2 removal might affect the LPS responses in macrophages.

To investigate whether and how the lack of GPS2 affects ATM function and gene expression *in vivo* under conditions of metaflammation, WT and MKO mice were subjected to 60% HFD feeding, compared to the low-fat diet (LFD) for 12 weeks. We found that removal of GPS2 from macrophages did not result in significant changes in body weight gain, and food and water intake (data not shown). Most strikingly, the infiltration of macrophages (F4/80+CD11b+ ATMs) was increased in MKO compared to WT mice within both epididymal white adipose tissue (epiWAT) and inguinal white adipose tissue (ingWAT), as visualized by immunohistochemistry (IHC) and quantified by flow cytometry. Importantly, analysis of *Gps2* expression in epiWAT of WT mice subjected to control LFD

versus HFD for 4, 8 and 12 weeks revealed a significant decrease of *Gps2* mRNA levels. Therefore, the down-regulation of *Gps2* levels in response to HFD might be a compensatory mechanism that limits the anti-inflammatory action of GPS2 in macrophages under conditions of obesity.

The intersection of the GPS2 cistrome, epigenome, and transcriptome.

In order to define the genomic DNA occupancy (the cistrome) of GPS2, in relation to the epigenetic landscape (the epigenome) and gene expression (the transcriptome), we performed ChIP-seq in BMDMs of GPS2 WT versus MKO mice. *De novo* motif analysis showed that GPS2-enriched regions were specifically bound for motifs recognized by LDTFs such as PU.1/ETS, C/EBP, and RUNX, which is consistent with the role of these factors in macrophages [159]. Amongst the motifs recognized by SDTFs, AP-1/ATF and IRF motifs were most abundantly enriched in GPS2-bound regions, suggesting the communication between GPS2 and these TFs to repress pro-inflammatory gene expression. Surprisingly, NF-κB motifs and nuclear receptor half-site motifs were only poorly enriched of the GPS-bound regions, raising questions about the importance of these TFs in communicating with GPS2 in macrophages, at least at the genome-wide level. The comparative analysis of changes in histone marks (epigenome) and transcriptome in MKO versus WT macrophages uncovered quantitative increases of H3K27ac and H3K4me3 signal intensity at enhancers/promoters of pro-inflammatory signature genes that were up-regulated upon GPS2-removal indicative of transcriptional de-repression/activation. As an example, in GPS2-deficient macrophages H3K27 acetylation increased by up to 69% and 109% at the promoters of the 'GPS2-sensitive' *Ccl2* and *Ccl7* gene cluster respectively, and by 65% at the first *Ccl2* enhancer E1, which was 35 kb upstream of transcription start site (TSS), and around 41% at the second *Ccl2* enhancer E2, which was 15 kb upstream of TSS.

Macrophage GPS2-deficiency causes enhanced pro-inflammatory gene expression in AT in response to HFD feeding.

To test whether increased macrophage infiltration in MKO AT was likely a consequence of corresponding changes in gene expression, MKO versus WT mice were subjected to HFD feeding and analyzed by microarray and qRT-PCR. This was further substantiated by a kinetic qRT-PCR analysis of the HFD response in AT, demonstrating an elevated expression of GPS2 signature chemokines, such as *Ccl2*, and cytokines, such as *Il6*, in both epiWAT and ingWAT of MKO mice. Consistent with the fact that visceral fat depots are

often more susceptible to inflammation than subcutaneous fat, the elevated pro-inflammatory signature in epiWAT was seen already after 4 weeks of HFD feeding. These changes were confirmed to specifically occur in the F4/80+ macrophage fraction, but not in adipocytes. To support that the changes were specifically caused by GPS2, and not by alterations of associated complex subunits, we validated that the expression of *Ncor* and *Smrt* was not changed in macrophages or adipocytes of epiWAT. We finally demonstrate that the transcriptome alterations in GPS2-deficient macrophages provoked a systemic increase of the encoded gene products CCL2, IL6, and TNFα upon HFD. Thus, macrophage GPS2 likely has a crucial role in limiting metaflammatory responses in the AT itself, and in limiting systemic responses originating from AT under conditions of diet-induced obesity.

Macrophage GPS2-deficiency exacerbates insulin resistance in peripheral tissues during diet-induced obesity.

In light of the initially observed correlation between GPS2 levels and insulin resistance score upon HFD feeding, we closer investigated glucose homeostasis in MKO versus WT mice. We observed that both fasting and feeding blood glucose levels were elevated in MKO mice, in conjunction with increased serum insulin levels, suggesting that these mice were more insulin-resistant according to the HOMA-IR index. Oral glucose tolerance test (OGTT) showed that MKO mice were less glucose-tolerant than WT mice, despite increased serum insulin during the glycemic burst. Insulin tolerance test (ITT) confirmed that MKO mice became more insulin resistant than WT mice after 12 weeks of HFD. For both tests, no changes between WT and MKO mice were detected under control LFD conditions.

Taken together, the MKO mouse model-derived results infer a mechanism thereby macrophage GPS2 protects against metabolic inflammation of AT, contributes to maintaining systemic insulin sensitivity, and prevents ectopic lipid partitioning in the liver (steatosis) under conditions of HFD-induced obesity. Overall, the findings of this paper suggest a causal relationship between GPS2 expression and function, the extent of tissue inflammation, and insulin resistance, which appears conserved in mice and humans (**Figure 11)**

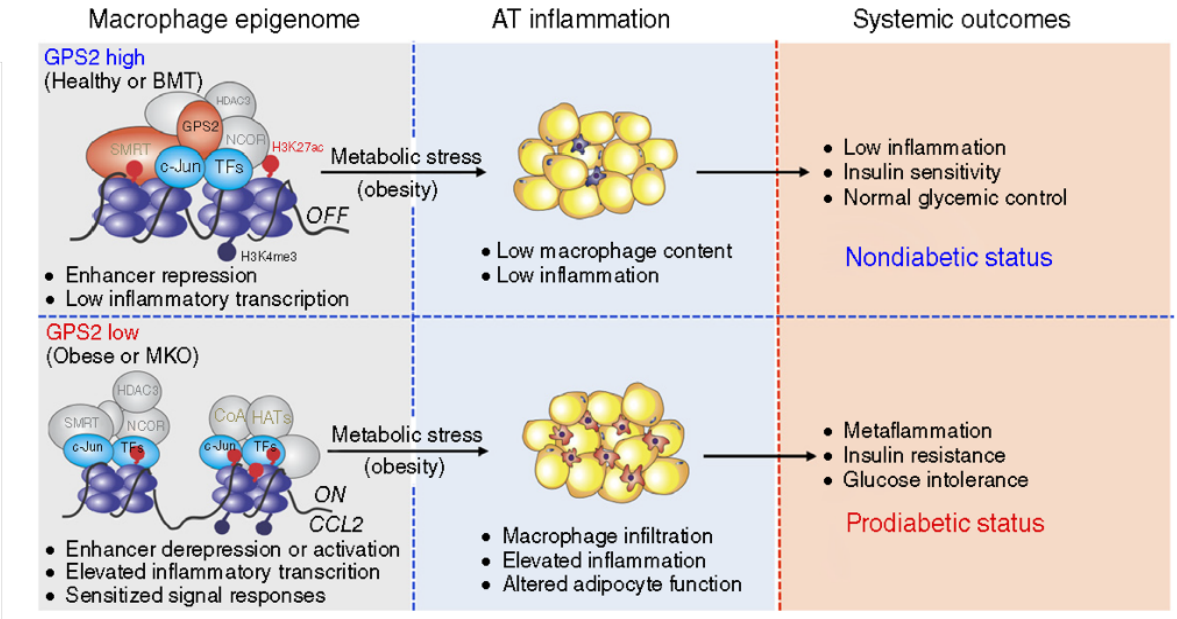

Figure 11. Model linking GPS2, epigenome alterations, and metaflammation.

Reprinted from Nature Medicine, Vol.22. p.780-791, Rongrong Fan et al., Loss of the co-repressor GPS2 sensitizes macrophage activation upon metabolic stress induced by obesity and type 2 diabetes, doi: 10.1038/nm.4114, Copyright 2016, with permission from Springer Nature.

4.4 Paper IV: Liver-specific loss of GPS2 reveals functions in lipid metabolism and NAFLD

Liver GPS2 expression is associated with fibrogenic gene expression in human NASH patients.

To explore the potential functions of GPS2 in liver steatosis and fibrosis, we first compared the expression of all GPS2 corepressor complex core subunits in non-alcoholic steatohepatitis (NASH) transcriptome data from human patients (in collaboration with Dr. Bart Staels, INSERM Lille). The analysis in the NASH subjects showed 66 genes were positively correlated with *GPS2* expression. Among them were fibrosis genes *TGFB, TIMP1, ACTA2, TIMP1*, and lipogenic gene *SCD1*. The further analysis showed this gene signature was correlated with *NCOR, TBL1, TBLR1*, and *HDAC3*, but not with *SMRT*. More datasets were used for the compression in the fibrosis stages, which show GPS2 expression was positively correlated with fibrosis genes, including *TGFB, TIMP1*, and *ACTA2*. *GPS2* expression level was high in NASH fibrosis than the non-fibrosis liver biopsies. Moreover, the *GPS2* level was restored after dietary intervention or gastric bypass surgery in the obese human subjects. In summary, the human data indicated that GPS2 might promote the liver fibrosis and contribute to obesity-associated glucose dysregulation.

Liver-specific Gps2 knockout in mice improves fibrosis and obesity-associated liver steatosis and insulin resistance.

We further investigated the causative function of GPS2 *in vivo* using hepatocyte-specific GPS2 knockout mice (LKO). By feeding with methionine- and choline-deficient diet (MCD), LKO mice showed improvement of liver fibrosis by significant reduction of serum aspartate transaminase (AST) and alanine transaminase (ALT) activity. GPS2 LKO mice showed improved obesity-associated liver steatosis and insulin resistance phenotype. When treated with a HFD, LKO mice body weight was significantly lower than the control group. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) showed improved insulin sensitivity and glucose control. Consistently, both serum VLDL, total triglyceride levels and intra-hepatic triglyceride levels were decreased in LKO mice. These results indicated that GPS2 in hepatocyte plays a crucial role in both lipid metabolism and glucose homeostasis.

PPARα is a direct target of GPS2 in the liver.

In order to explore mechanisms involved in the regulatory role of GPS2 in the liver, we analyzed the GPS2 LKO RNA-seq data and identified PPARα pathways target genes were enriched by GPS2 depletion. We further performed the GPS2 ChIP-seq to obtain cistrome profiles in the liver. Consistent with the transcriptome data, TF binding motif analysis revealed PPAR binding sites among the top GPS2-occupied sites. To test the requirement of PPARα for the GPS2 repression of lipid metabolic genes, we generated GPS2 and PPAR*α* double KO (PGKO) mice. The elevated lipid oxidation observed in LKO mice was not observed in PGKO mice, as indicated by unchanged fed and fasted ketone body generation, which suggests that GPS2 repression of lipid oxidation was dependent on PPAR*α*. Consistently, qRT-PCR showed that GPS2 ablation did not induce the PPARα target genes *Pdk4*, *Cyp4a14*, *Fgf21* in PGKO livers. Collectively, these data identify PPARα as a likely target TF for GPS2 in the liver.

GPS2 cooperates with NCOR to modulate liver epigenome and transcriptome.

To define the GPS2-dependent cistrome and epigenome in mouse liver, we performed ChIP-seq of GPS2 along with H3K27ac and H3K4me3 in WT and LKO mice. Comparison of the GPS2-dependent transcriptome (RNA-Seq) and epigenome (H3K27ac and H3K4me3) revealed that transcriptional and epigenetic activation (i.e., changes in activating histone modifications) at GPS2-sensitive gene loci are highly coordinated. Consistent with the lack

of gene expression changes, H3K27ac levels at GPS2-sensitive gene loci were similar in PGKO mice compared to PKO mice, which indicated the epigenetic activation of these genes upon GPS2 depletion was dependent on PPARα. We further investigated whether GPS2 functions within the corepressor complex to modulate liver gene expression. Cistrome analysis revealed that GPS2, NCOR, SMRT are present at promoters and enhancers of PPARα target genes including *Pdk4* and *Cyp4a14*. However, KEGG pathway analysis of the genes co-repressed by GPS2 and NCOR revealed PPAR signaling in the top list, along with liver metabolic pathways, which was not observed for other genes co-repressed by GPS2 and SMRT. In sum, the above data suggest that GPS2 repression of PPARα target genes in hepatocytes involves functional cooperation with NCOR, while the loss of GPS2 in LKO mice results in epigenome remodeling of the corresponding promoter/enhancer regions leading to PPARα target gene activation.

In conclusion, this study identifies GPS2 as an epigenome modifier and PPARα corepressor in hepatocytes that inhibition has the therapeutic potential to reverse the progression of NASH toward fibrosis. The proposed link of GPS2 to NCOR in hepatocytes raises questions as to the different corresponding KO phenotypes (**Figure 12**). While *Gps2* LKO resulted in improved liver steatosis and reduced lipid accumulation, NCOR LKO resulted in the opposite, i.e., increased lipid accumulation [160, 161].

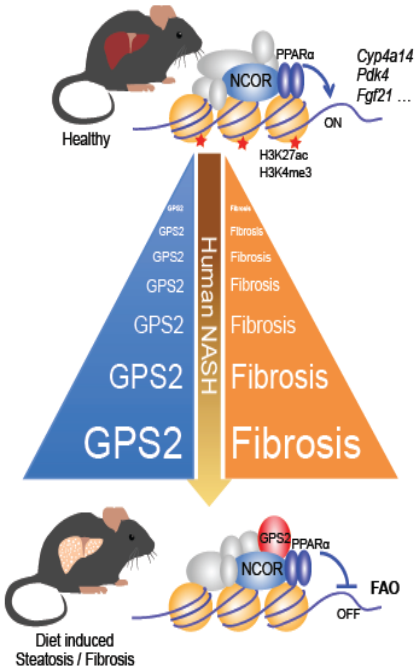

Figure 12. Model highlighting the putative role of GPS2 in fatty liver disease.

5 CONCLUDING REMARKS AND PERSPECTIVES

The four studies in this thesis have focused on the roles of GPS2 in obesity/T2D-related inflammation and lipid/cholesterol metabolism, which elaborated potential GPS2-mediated mechanisms that link epigenome alterations and metabolic disease. To this end, this thesis used both *in vitro* and *in vivo* models to investigate the function of GPS2 in closely linked inflammatory and metabolic signaling pathways. The *in vitro* studies not only enabled us to verify and test potential mechanisms underlying the phenotypes of the *Gps2* knockout mice but they also resulted in new complementary findings that would have been difficult to detect *in vivo*.

The probably most critical finding in this thesis is that GPS2 inhibits the transcription of crucial pro-inflammatory enhancers and genes such as *Ccl2* in macrophages. Thereby, GPS2 plays pivotal roles in counteracting inflammation in the context of obesity and T2D. Intriguing mechanistic results of this thesis are that this anti-inflammatory function of GPS2 in macrophages requires cooperation with SMRT, while the metabolic function in the liver requires cooperation with NCOR. Although the reasons for this cell type-selective cooperativity are currently unknown, the data suggest that GPS2 functions within corepressor sub-complexes, a hitherto unrecognized feature of this essential epigenome modifier [66]. Additionally, GPS2 also works 'out of complex' to induce ABCA1 expression linked to cholesterol efflux from macrophages, indicating that there is a fraction of 'free' GPS2 involved in repression-independent transcriptional regulation.

The key results of this thesis may help to better understand mechanisms underlying the development, prevention and treatment of metaflammatory diseases, in particular, obesity-associated T2D and atherosclerosis.

In **Study I**, we demonstrate that GPS2 displays a unique function in controlling endotoxin (LPS)-induced macrophage cholesterol efflux via ABCA1. We also provide evidence that the GPS2-ABCA1 pathway is conserved in mouse and human macrophages. Thus, the study contributes to better understanding the macrophage-mediated pathways that link obesity-associated inflammation, T2D, and cholesterol efflux to the development of cardiovascular diseases. More specifically, our results should help to re-interpret the

involvement of GPS2 and ABCA1 in mechanisms of anti-inflammatory trans-repression by LXRs [82, 162-166]. This may stimulate the future investigation of anti-inflammatory drug candidates that modulate the GPS2-ABCA1-LXR axis.

In **Study II**, we uncover molecular features of GPS2-repressed inflammatory enhancers, with a particular emphasis on the *Ccl2* super-enhancer that controls metaflammation both locally in the adipose tissue and systemically. Macrophage-derived CCL2 is a major chemokine and drug target involved in recruiting immune cells to metabolic tissues, leading to peripheral insulin resistance [36, 167]. We additionally provide evidence that enhancer-transcribed eRNAs may have a function, which would be a significant advance in the field. Future efforts should attempt to specifically target eRNAs via antisense LNAs *in viv*o, for example in obese mouse models. This would provide *in vivo* validation of eRNA function and describe pre-clinical models for future eRNA-targeted therapies. Indeed, the feasibility of targeting tissue-specific and disease-relevant enhancers using LNA or CRISPR-based strategies might have clinical importance [168, 169].

In **Study III**, we demonstrate that macrophage-specific *Gps2* knockout mice display the hallmarks of metaflammation typical for humans with obesity and/or T2D. The *in vivo* mouse model and the *in vitro* studies using isolated macrophages provide strong support for the suspected causal role of GPS2 dysregulation in driving epigenome alterations linked to metaflammation in humans. The underlying mechanisms identified in this study suggest therapeutic possibilities for delaying or reversing obesity/T2D-related inflammation through the restoration of GPS2 expression and function. Although GPS2 protein itself is currently unlikely to be a feasible target for drug development, the modulation of protein-protein interactions or of post-translational modifications may offer alternatives.

In **Study IV**, we show that liver-specific *Gps2* knockout in mice improves obesity-associated liver steatosis and insulin resistance. These results, along with expression correlation analysis in human liver samples and the identification of PPARα as target TF, suggest that the modulation of the GPS2-PPARα network could help to restore altered lipid metabolism and glucose homeostasis in NAFLD.

Overall, this thesis puts forward the concept of a ‘GPS2 dysfunction-epigenome alterations-metaflammatory disease’ axis, which should be further employed mechanistically and therapeutically in different cell types and signaling contexts.

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